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ORIGINAL ARTICLE

Influenza A virus drift variants reduced the detection sensitivity of a commercial multiplex nucleic acid amplification assay in the season 2014/15

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Abstract The influenza season 2014/15 was dominated by drift variants of influenza A(H3N2), which resulted in a reduced vaccine effectiveness. It was not clear if the performance of commercial nucleic-acid-based amplification (NAT) assays for the detection of influenza was affected. The purpose of this study was to perform a real-life evaluation of two commercial NAT assays. During January-April 2015, we tested a total of 665 samples from patients with influenza-like illness using the Fast Track Diagnostics Respiratory pathogens 21, a commercial multiplex kit, (cohorts 1 and 2, n = 563 patients) and the Xpert Flu/RSV XC assay (cohort 3, n = 102 patients), a single-use cartridge system. An in-house influenza real-time RT-PCR (cohort 1) and the RealStar Influenza RT-PCR 1.0 Kit (cohort 2 and 3) served as reference tests. Compared to the reference assay, an overall agreement of 95.9 % (cohort 1), 95 % (cohort 2), and 98 % (cohort 3) was achieved. A total of 24 false-negative results were observed using the Fast Track Diagnostics Respiratory pathogens 21 kit. No false-negative results occurred using the Xpert Flu/RSV XC assay. The Fast Track Diagnostics Respiratory pathogens 21 kit and the Xpert Flu/RSV XC assay had sensitivities of 90.7 % and 100 % and specificities of 100 % and 94.1 %, respectively, compared to the RealStar 1.0 kit. Upon modification of the Fast Track Diagnostics Respiratory pathogens 21 kit, the sensitivity increased to 97.3 %. Influenza virus strains circulating during the 2014/15 season reduced the detection sensitivity of a commercial NAT assay, and continuous monitoring of test performance is therefore necessary.

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Introduction

Seasonal influenza in Europe in 2014/15 was dominated by drift variants of influenza A(H3N2) virus [1, 2]. Critically, these variants resulted in reduced influenza vaccine effectiveness [3]. From a laboratory perspective, recent experience with influenza virus variants showed that the performance of diagnostic methods can be affected [4, 5]. However, a reliable diagnosis is of utmost importance for patient management, hospital hygiene, and public health. Nucleic acid amplification tests (NATs) are considered the gold standard for the laboratory diagnosis of influenza, and a number of commercial and in-house methods are available. Importantly, the genetic drift and shift of influenza A viruses requires continuous monitoring and update of NAT assays. Although the matrix and nucleoprotein genes of influenza virus are preferred target regions of molecular assays due to their rather conserved nature, they also evolve over time [6]. Previous studies have shown the effect of mutations, e.g., in the matrix gene of influenza virus, which reduced the detection sensitivity of in-house and commercial NAT assays [7, 8]. Of note, commercial companies are sometimes constrained in their ability to modify assays quickly due to regulatory issues.

Soon after the beginning of the 2014/15 influenza season, we observed two false-negative results using a commercial multiplex assay for detection of influenza virus. In both cases, the clinical suspicion of influenza remained high. Therefore, an additional in-house influenza real-time RT-PCR was performed, which yielded positive results, whereupon all respiratory samples were prospectively tested in parallel using the multiplex PCR and the influenza in-house RT-PCR. To systematically address the performance of the multiplex assay, retrospective data were collected at a second study site. In addition, a common commercial cartridge-based influenza NAT assay was retrospectively evaluated.

In this paper, we performed a real-life evaluation of two commercial NAT assays.

Materials and methods

Study cohorts

A total of 665 clinical samples submitted for routine influenza diagnostics at two study sites between January and April 2015 were included. Three cohorts were analysed: in cohorts 1 and 2 the Fast Track Diagnostics Respiratory pathogens 21 kit (FTD21; Fast Track Diagnostics, Junglingster, Luxemburg), a broad-range multiplex NAT, was used as the first-line test. In cohort 3, Xpert Flu/RSV XC (Xpert; Cepheid, Frankfurt, Germany), a single-use cartridge system, was used as the first-line test. The choice of assay was done at the discretion of the laboratory and was based on daily workload, working hours, and urgency for results.

Cohort 1 (n = 413) included samples submitted to the Institute of Clinical and Molecular Virology in Erlangen, Germany. All samples of cohort 1 were prospectively analysed in parallel with an influenza in-house real-time RT-PCR [9, 10].

Cohort 2 (n = 150) included 69 influenza-positive samples (32 influenza A(H3N2), 15 influenza A(H1N1)pdm09, 22 influenza B), and 81/150 (54 %) influenza-negative samples using the FTD21 kit at the Institute of Virology, Freiburg. The median age was 62 years (range, 1 month to 95 years). Samples included 144 pharyngeal swabs in viral transport medium (UTM, Copan, Italy), two nasopharyngeal secretions, and four broncho-alveolar lavage samples.

Cohort 3 (n = 102) included 69 influenza-positive samples (44 influenza A and 25 influenza B), and 33 samples that were influenza negative when using the Xpert assay at the Institute of Virology, Freiburg. The median age was 70 years (range 6 months to 92 years). Samples included 100 pharyngeal swabs in viral transport medium (UTM, Copan, Italy), one nasopharyngeal secretion, and one broncho-alveolar lavage sample. Cohorts 2 and 3 comprised randomly selected samples from routine diagnostics, which were retrospectively analysed using a RealStar Influenza RT-PCR 1.0 kit (RealStar 1.0; Altona Diagnostics, Hamburg, Germany). The RealStar 1.0 kit served as the reference assay for cohorts 2 and 3. The decision to retrospectively analyze samples at the Freiburg study site was made within the ongoing influenza season when sensitivity problems with the FTD21 assay were communicated. Further, including two study sites minimized a possible influence of differences in the circulating influenza subtypes from different geographical regions.

Virological analysis

For cohort 1, nucleic acid preparation was done using an EZ1 Virus Mini kit (QIAGEN) on an EZ1 XL instrument (QIAGEN). A sample input volume of 200 µl was used and the elution volume was set to 90 µl. The FTD21 kit in combination with an AgPath-ID One-Step RT-PCR Kit (Thermo Fisher, Darmstadt, Germany) was used on an Applied Biosystems 7500 cycler (Thermo Fisher) according to the manufacturer's instructions. The cycling profile was 50 °C for 15 min and 95 °C for 10 min, followed by 40 cycles of 95 °C for 8 s and 60 °C for 34 s. The FTD21 kit is able to detect influenza A and B viruses and allows detection of the A(H1N1)pdm09 hemagglutinin gene. Samples that were positive in the general influenza A assay but negative for A(H1N1)pdm09 were classified as A(H3N2) based on the current epidemiological situation in Germany. For laboratory confirmation, a random selection of samples were typed using an A(H3N2)-specific real-time RT-PCR as described (protocol available upon request) [11]. Influenza in-house real-time RT-PCR was adapted from the literature and done as described (Electronic Supplementary File 1) [9, 10]. Samples that were positive in the influenza in-house real-time RT-PCR but negative in the FTD21 assay were further analysed for the HA genes of A(H1N1)pdm09 according to Panning et al., and seasonal A(H3N2) viruses were analyzed according to Schulze et al. [4, 11]. The detection of circulating influenza A(H3N2) virus from 2015 was verified on samples kindly supplied by the German National Reference Center for Influenza (data not shown).

For cohort 2, sample preparation was done using a QIAmp MinElute Kit on a QIAcube instrument (QIAGEN, Hilden, Germany). The sample input volume was 200 µl, and the elution volume was 100 µl. The FTD21 kit was used as described for cohort 1. In April 2015, a modified (influenza primer/probe mix) version of the FTD21 kit (FTD21mod) became available. The FTD21mod was accompanied by an enzyme blend supplied by the

manufacturer, and a subset of 30 randomly selected influenza-positive samples based on availability was re-tested using FTD21mod. The following cycling profile was used: 42 °C for 15 min, and 94 °C for 3 min, followed by 40 cycles of 94 °C for 8 s and 60 °C for 34 s. Nucleic acid preparations were stored at -20 °C and re-tested in August 2015 using the RealStar 1.0 kit as recommended by the manufacturer. This assay detects and differentiates influenza A and B viruses. Verification of the RealStar 1.0 to detect contemporary A(H3N2) samples was done successfully (data not shown).

For cohort 3, samples were tested immediately upon receipt using the Xpert Flu/RSV XC assay according the manufacturer's recommendation. Leftover original material was frozen at -20 °C until August 2015. Samples were extracted using a MagnaPure Total Nucleic Acid Isolation Kit on a MagnaPure LC 2.0 instrument (Roche, Mannheim, Germany) with 200-µl sample input and 100-µl elution volume. The MagnaPure was chosen due to its high-throughput capability and high degree of automation. The extraction efficiency was comparable to that of the QIAmp MinElute Kit (own unpublished observation). Samples were tested using the RealStar 1.0 kit. For typing all Xpert-positive influenza A samples, the Fast Track Diagnostics Flu differentiation kit was used according to the manufacturer's instructions using the same nucleic acid preparation. Additionally, the RealStar Influenza S&T RT-PCR 3.0 kit was used to analyse discrepant influenza B samples according to the manufactureŕs instructions.

Statistical analysis

Sensitivity, specificity, and kappa were calculated using GraphPad Prism 6 (GraphPad software, USA). Median Ct values between two groups were compared using the Wilcoxon rank sum test. *P*-values ≤ 0.05 were considered statistically significant.

Results

Cohort 1: FTD 21 versus in-house RT-PCR

In cohort 1, a total of 413 samples from 367 patients were analysed. The median age of the 367 patients was 59.2 years (range, 5 weeks to 91 years). Samples included 92 pharyngeal swabs, 177 throat washings, 30 tracheal secretions, 96 bronchoalveolar samples, and 18 other respiratory samples.

Using the FTD21 kit, 33 of 413 (7.9 %) samples tested positive for influenza A(H3N2) virus, 12 of 413 (2.9 %) for influenza A(H1N1)pdm09 virus, and 19 of 413 (4.6 %) for influenza B virus, whereas 349 of 413 (84.5 %) samples tested negative for influenza A and B virus. A total of 17 randomly selected A(H3N2)-positive samples were also positive using an A(H3N2)-specific real-time RT-PCR, thus confirming the presence of A(H3N2) virus. All 64 samples that tested positive for influenza A or B virus RNA in the FTD21 assay were also positive in the corresponding influenza in-house real-time RT-PCR. The median Ct values of influenza A(H3N2)-, A(H1N1)pdm09-, and B-positive samples were not significantly different between the FTD21 assay and the in-house assay (data not shown). Among the 349 FTD21 influenza-negative samples, 17 (4.9 %) tested positive using the in-house assay. Of these, 16 of 17 (94 %) were positive for influenza A(H3N2) virus (all confirmed positive using A(H3N2)-specific real-time RT-PCR) and one of 17 (6 %) was positive for A(H1N1)pdm09. The median Ct value of these A(H3N2)-positive samples was 33 (interquartile range [IQR] 30.3-35.8) compared to a median Ct value of 27.3 [IQR 24.3-30.2] for the A(H3N2) samples that were positive in both assays, indicating low virus concentrations in the discrepant samples. The Ct value of the A(H1N1)pdm09-positive sample was 36. Overall, agreement between the FTD21 and the in-house assay for detection of influenza virus was 95.8 %.

Cohort 2: FTD 21 versus RealStar 1.0

In cohort 2, all 69 samples that were positive for influenza A or influenza B virus using the FTD21 assay also tested positive using the RealStar 1.0 kit. The median Ct values for each subtype were significantly higher in samples using the FTD21 kit compared with the RealStar 1.0 kit (Fig. 1A). Among the 81 samples that tested negative using the FTD21 kit, the RealStar 1.0 kit detected 7 of 81 (8.6 %) influenza-positive samples (six influenza A and one influenza B). The median Ct-value of these seven samples was 36 (IQR 32-36), again indicating a low virus concentration. Thus, an overall agreement of 95.3 % was achieved between the FTD21 kit and the RealStar 1.0 kit. In the next step, the FTD21mod kit was used to re-analyze the seven discrepant samples. Six of these seven samples now tested positive for influenza virus (five influenza A(H3N2) and one influenza B). The median Ct value of the six influenza-positive samples using FTD21mod compared with the RealStar kit was not significantly different (data not shown). Finally, a subset of influenza-positive samples ($n = 30$) was retested using the FTD21mod kit, and Ct values were compared with the RealStar 1.0 kit. Median Ct values for influenza A(H3N2) and B viruses were not statistically different, but Ct values for A(H1N1)pdm09 were significantly lower using the FTD21mod kit (Electronic Supplementary File 2).

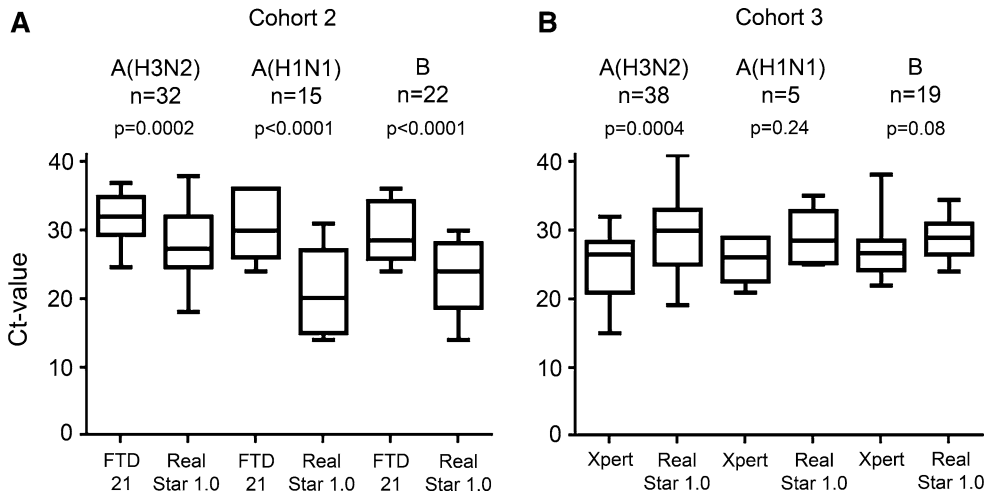

Fig. 1 **A.** Comparison of Ct values of samples positive for A(H3N2), A(H1N1)pdm09 (A(H1N1)), and B viruses using the FTD21 and the RealStar 1.0 kit, respectively (cohort 2). The center line within each boxplot represents the median Ct value; the box, the interquartile range; and the whiskers, the minimum and maximum Ct values. The number positive for each subtype is given below. *P*-values are given for differences between Ct values measured using the FTD21 kit and the RealStar kit, respectively. **B.** Comparison of Ct values of samples positive for A(H3N2), A(H1N1)pdm09 (A(H1N1)), and B viruses using the Xpert kit and the RealStar 1.0 kit, respectively (cohort 3). The center line within each boxplot represents the median Ct value; the box, the interquartile range; and the whiskers, the minimum and maximum Ct values. The number positive for each subtype is given below. *P*-values are given for differences between Ct values measured using the Xpert kit and the RealStar kit, respectively

Cohort 1 and 2 combined included 563 samples, of which 157 were positive for influenza virus when using the reference assay (in-house assay or RealStar 1.0 kit). A total of 24 of 157 (15 %) of these influenza-positive samples were missed using the FTD21 kit.

Cohort 3: Xpert versus RealStar 1.0

In cohort 3, a total of 67 of 69 (97.1 %) Xpert-positive samples yielded results that were concordant with those of the RealStar 1.0 kit (43/44 influenza A and 24/25 influenza B). To determine the influenza A virus subtype in cohort 3, a Fast Track Diagnostics Flu differentiation kit was used (A(H3N2), n = 38; A(H1N1)pdm09, n = 5). For the A(H3N2)-positive samples, the median Ct values of the Xpert assay were significantly lower compared to the RealStar 1.0 assay (Fig. 1B).

The two discrepant samples had Ct values of 34 (influenza A) and 33 (influenza B), respectively, in the Xpert assay. The influenza A sample that was positive only in the Xpert assay remained negative using the Fast Track Diagnostics Flu differentiation kit. The influenza B sample that was positive only in the Xpert assay also remained negative using the RealStar Influenza S&T RT-PCR 3.0 Kit. Therefore, these two samples were considered false-positives in the Xpert assay. All 33 Xpert-negative samples tested negative using the RealStar 1.0 kit. Overall, agreement between both assays was 98 %.

Finally, the performance of the FTD21 kit and the Xpert assay compared to the RealStar 1.0 kit for influenza A and B combined was calculated. The FTD21 kit and the Xpert assay had sensitivities of 90.7 % and 100 % and specificities of 100 % and 94.1 %, respectively (Table 1). Sensitivity increased to 97.3 % using the FTD21mod kit.

Discussion

Here, we show that a total of 24 of 157 (15 %) influenza-positive samples were missed by the FTD21 kit, supporting the notion that circulating influenza A virus strains in 2015 had an impact on assay performance. A similar finding using another commercial multiplex PCR was recently reported in Belgium [12]. Intriguingly, false-negative results using the FTD21 assay were observed at both study sites (cohort 1 and 2). This finding points towards a generalized problem rather than technical issues in a single laboratory. We used two independent reference assays that have demonstrated excellent diagnostic performance [10, 13]. In addition, both reference assays were capable of detecting influenza A(H3N2) virus drift variants of 2015 (our own unpublished observation). A limitation of our study, it should be noted, is that the extraction methods used were slightly different due to the real-life setting of our study. However, the sample input and elution volumes essentially remained the same, and no significant differ-

Table 1 Sensitivity specificity, and kappa value for the FTD21 kit, FTD21mod kit (sensitivity only), and the Xpert assay in comparison to the RealStar 1.0 kit

	TP	TN	FP	FN	% Sensitivity (95 % CI)	% Specificity (95 % CI)	kappa
FTD21	69	74	0	7	90.7 (81.3-95.9)	100 (93.9-100)	0.91
FTD21mod	36	-	-	1	97.3 (84.2-99.9)	na*	na*
Xpert	67	33	2	0	100 (93.2-100)	94.3 (79.4-99)	0.98

TP, true positive; TN, true negative; FP, false positive; FN, false negative

* na = not applicable

ences in extraction efficiency were noted. In addition, absolute quantification might provide a better resolution of the assay performance than using Ct values [13]. Apart from technical issues, it is important to note that patient management and public health strongly depend on the correct diagnosis. It remains speculative if the false-negative results had a detrimental effect on patient outcome.

Importantly, the sensitivity of the FTD21 kit significantly improved upon modification of the assay by the manufacturer. This became evident by the resolution of all but one discrepant result in comparison to the RealStar 1.0 reference assay. Interestingly, an improvement with respect to Ct values was also observed for the detection of A(H1N1). The proprietary nature of the primer/probe sequences prevented a deeper investigation. Thus, it remains speculative if a modified primer/probe mix, a novel enzyme with a lower temperature profile for the reverse transcription step, or a combination of both accounted for the improvement. Of note, the modified FTD21 kit became available soon after the identification of the reduced test sensitivity while maintaining regulatory approval.

Testing a large panel of clinical samples, we were able to demonstrate a good overall agreement between the FTD21 assay and a validated in-house RT-PCR and the RealStar 1.0 assay, respectively. This finding is reassuring, showing that a commonly used multiplex NAT kit allowed the detection of influenza virus in a season that was dominated by drifted influenza A(H3N2) and B strains. In Germany, all characterized influenza A(H3N2) viruses were antigenically similar to A/Switzerland/9715293/2013, which was considered a drift variant when compared to A/Texas/20/2012 [2]. Importantly, this drift variant was also the dominant influenza virus in other parts of Europe [1]. We did not systematically sequence all influenza-A-virus-positive samples, but the presence of an A/Switzerland/9715293/2013-like virus in a limited number of samples from Freiburg was confirmed at the German National Reference Center for Influenza. The high specificity of the FTD21 kit has been demonstrated before and was confirmed in our study [14].

In addition, we were able to confirm the high sensitivity and specificity of the Xpert Flu/RSV XC assay, although specificity in our study was slightly lower than reported [15, 16]. It is noteworthy that in both of these studies, different laboratory-developed RT-PCR assays were used as reference assays, thus reducing the comparability of the studies. Although we used a commercial NAT assay as a reference, failure of our reference assay cannot be ruled out with certainty. Interestingly, our reference assay showed significantly higher Ct values for A(H3N2) compared to the Xpert assay, but not for A(H1N1) and B, suggesting a limited influence of currently circulating strains on the performance of the RealStar 1.0 kit. Again, proprietary issues prevented a deeper investigation of the primer/probe regions. In this respect, customers of commercial kits are constrained in their response to newly emerging variants and have to rely on the manufacturer. Knowledge of the primer and probe sequences is clearly an important issue for diagnostic laboratories. Rapid modification of primers and probes can compensate for newly emerging mutations and restore sensitivity as demonstrated for the influenza A(H1N1)pdm09 virus [17]. On the other hand, with respect to standardization and quality control of reagents, commercial assays are preferable [13, 18].

Due to their high sensitivity and specificity, NAT assays are the gold standard in detection of influenza virus. Recent advances in the field include multiplex NAT panels for the simultaneous detection of a broad range of pathogens and cartridge-based systems with a short turnaround time. Importantly, a high diagnostic accuracy of these systems has been shown [16, 19]. However, due to the rapid evolution of influenza viruses new variants might carry mutations in the primer- and probe-binding sites of NAT assays, which can have a detrimental effect on the sensitivity of assays [20]. In the light of recently published studies and our data, it is evident that backup assays are necessary in diagnostic laboratories to rapidly respond to sensitivity issues.

The fast evolution of influenza viruses demands constant monitoring of the evolution of influenza virus worldwide. This allows detection and anticipation of epidemiological

trends that are important for vaccine production as well as development of diagnostic tests. For diagnostic purposes, targeting multiple genes simultaneously is an option to increase the reliability of NAT assays to detect influenza virus. Alternatively, the use of degenerate oligonucleotides that tolerate mismatches better might prevent false-negative results.

Conclusion

The influenza season 2015 had an impact on the performance of commercial NAT assays, and continuous evaluation of assays seems to be important. Clinical laboratories and companies should be aware that mutations within the target region of NAT assays can evolve, allowing variant strains to go undetected.

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Compliance with ethical standards

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Competing interests None declared.

Ethical approval Not required.

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